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Development of Laccase-TiO_2@Carbon Paste Biosensor for Voltammetric Determination of Paracetamol

Douglas Vieira Thomaz, Mayk Teles de Oliveira[*]*, Germán Sanz Lobón, Carlos Eduardo Peixoto da Cunha, Fabio Bahls Machado, Emily Kussmaul Gonçalves Moreno, Karla Carneiro de Siqueira Leite, Nara Ballaminut, Morgana Fernandes Alecrim, Murilo Ferreira de Carvalho, Bruna Guimarães Isecke, Isaac Yves Lopes de Macêdo, Renê Oliveira do Couto, Edson Silvio Batista Rodrigues, Luiz Alcir de Faria Carvalho, Leo Fernandes Ávila*

Faculdade de Farmácia, Universidade Federal de Goiás, Rua 240, S/n - Setor Leste Universitário, Goiânia - GO, 74605-170, Brazil.
[*]E-mail: mayk.teles@hotmail.com

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In this work a nanostructured TiO_2/carbon graphite matrix was employed in the development of a laccase (Lcc) carbon paste biosensor. The biosensor, hereby named Lcc-TiO_2@CPE had its response evaluated for paracetamol. Results evidenced that the biosensor was remarkably selective and sensible concerning paracetamol pharmaceutical form assessment. Linear response was obtained by differential pulse voltammetry (DPV) from 8 to 120 μM, with a limit of detection (LoD) of 1 μM, which was nine-fold superior than the one observed for unmodified carbon paste biosensor. Therefore, Lcc-TiO_2@CPE may be a novel low cost tool in analgesic drugs assessment.

Keywords: nanostructured electrodes; laccase; anti-inflammatory drugs; paracetamol; aspirin; pharmaceutical.

1. INTRODUCTION

Electro-analytical methods are highly regarded due to their economy in reagents and low cost, which combined to selectivity; sensibility and broad applicability, turns them into a useful strategy for the quality control assessment of drugs. Concerning such field, it is noteworthy that most excipients commonly employed in pharmaceutical technology are not electro-active, which in turn, increases the appeal of electro-analysis in dosage form determinations. Moreover, both sensibility and selectivity can be adjusted according to sensor composition [1,2,3].

The attractiveness of electrochemistry under the light of pharmaceutical analysis is often impaired by recurrent surface fouling on electrodes, which leads to low reproducibility. Henceforth, electrode surface renewal is one of the main goals in pharmaceutical electro-analysis. In this context, the use of carbon paste electrodes (CPE) offer the possibility to renew electrode surface between each assay, allowing therefore better inter and intra assay precision.

CPEs can be easily modified with a plethora of materials, such as nanostructured metal oxides, which allows enhanced performance [1]. Since electrode fouling occurs mostly at higher anodic potentials, any modifying agent capable of providing reproducible response at lower potentials would minimize electrode fouling, therefore improving measurement repeatability.

The use of biological recognizing agents combines the sensibility of electrochemical transducers with the selectivity of biochemical docking, therefore configuring biosensor technology as a powerful alternative towards drug assessment. Nonetheless, selectivity can be further enhanced by synergistic contribution of electrochemical and biological systems inherent specificity [4,5,6].

Amongst biological devices, enzymes are noteworthy due to their fast response, high reproducibility and reasonable stability [7,8]. Henceforth, many oxidoreductases and hydrolases are used as recognizing agents in amperometric and potentiometric biosensors [8,9]. Concerning oxidase-based biosensors, i.e. Laccase (Lcc), the oxidation step is biochemically controlled, thus avoiding high anodic potentials and hindering the fouling of the electrode [10].

Electroactive surface modification by nanostructured inorganic materials is a promising strategy to be explored in biosensor development [11,12,13], due to the synergistic effect promoted by metal oxide and enzyme association. Therefore, the aim of this paper was the development of a novel CP based biosensor capable of combining the outstanding biochemical performance of Lcc with TiO_2@C nanostructured materials. In order to evaluate the performance of the herein proposed biosensor, worldwide consumed phenolic drug, parecetamol, was employed as model.

2. EXPERIMENTAL

2.1. Reagents and solutions

Potassium ferrocyanide, ascorbic acid and catechol were purchased from Vetec Química Fina Ltda. (Rio de Janeiro, Brasil). Mineral oil and Titanium IV Isopropoxide were purchased from (Sigma-Aldrich, St. Louis, MO, USA). Milan® B (∅ = 5.2 mm) pencil graphite sticks, herein used to obtain modified graphite powder were purchased from a local store.

Salycilic acid and paracetamol were donated by the Pharmacy School Drugstore of Federal University of Goias, Goiânia-GO, Brazil. All standard solutions were prepared to render 1 mM stock solutions.

Electrolyte solutions were prepared using analytical grade reagents purchased from Vetec Química Fina Ltda. (Rio de Janeiro, Brasil), and diluted in purified water (conductivity ≤ 0.1 $\mu S.cm^{-1}$) obtained from Milli-Q purification system Millipore S/A (Molsheim, França).

2.2. Synthesis and characterization of modified pencil graphite powder (TiO_2@C)

Unmodified pencil graphite powder was produced by milling 1.5 g pencil graphite during 7 minutes at 20°C. Milling was conducted in a high energy planetary ball mill (Emax PM 100, Retsch®, Germany) at 650 rpm.

To produce TiO_2@C, 1.5g of unmodified pencil graphite powder was immersed and rigourosly mixed in 30 mL of ethanol:acetone solution (1:1) at 30°C for 10 minutes. Thereafter, 10 mL of ethanol:acetone solution (1:1) containing 1.3 % titanium IV isopropoxide was added to graphite suspension drop by drop during two hours. All aforementioned procedures were conducted at 30°C. The material was then dried in vacuum desiccator at 70ºC (Selecta heated vacuum desiccator "Vacuo-Temp", Spain).

The characterization of the resulting material was performed by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) experiments conducted in a JEOL Ltd. (Musashino, Akishima, Tokyo, Japan) JSM-6610 model and JEM-1400 model respectively. The magnification range was 1.000 to 15.000, with an accelerating voltage of 15.0 kV. The elemental analysis was performed by means of energy-dispersive X-ray (EDX).

2.3. Enzymatic crude extract

Lcc used to make the biosensor was obtained from *Pycnoporus sanguineus* cultures induced with xylidine and cooper sulfate. Lcc activity was of 401.20 U/mL, and determined by ABTS (diammonium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) [14].

2.4. Preparation of sensors and biosensors

Table 1. Sensors and biosensor composition.

Sensor material	Graphite powder	Nanoestructured $TiO_{2@}$C modified graphite powder	Laccase crude extract	Mineral oil
CPE	100 mg	-	-	30 mg
TiO_2@CPE	50 mg	50 mg	-	30 mg
Lcc-TiO_2@CPE	50 mg	50 mg	100 µL	30 mg

CPE was prepared as control, by mixing 100 mg of unmodified graphite powder with 30 mg of mineral oil (Nujol®), herein used as agglutinating agent.

TiO_2@CPE was prepared by mixing 50 mg of unmodified graphite powder and 50 mg of TiO_2@C with 30 mg of mineral oil.

Biosensor (Lcc-TiO2@CPE) was prepared by addition of 100 µL of Lcc extract to unmodified graphite powder. The resulting material was dried prior oil addition.

Each aforementioned material was homogenized and used to fill up a 6.28 mm^3 cavity on the supporting electrode.

The proportions employed for each electrode are described bellow in Table 1

2.5. Electrochemical assays

Electrochemical impedance spectroscopy (EIS) and Differential Pulse Voltammetry (DPV) measurements were performed using a potentiostat/galvanostat PGSTAT® model 204 with FRA32M module (MetrohAutolab) integrated with NOVA 2.1® software. All measurements were performed in 5 mL one-compartment electrochemical cell coupled to a three-electrode system consisting of the working electrodes described in Table 1, Pt wire and Ag/AgCl/KCl_{sat} (both purchased from Lab solutions, São Paulo, Brazil). The electrodes cited above represent working, counter and reference electrode, respectively.

EIS measurements were conducted in solutions containing 0.05 M potassium ferrocyanide in 0.1 M KCl over a frequency ranging from 0.01 Hz a 100 KHz at selected potentials for both the employed biosensor and sensors.

The experimental conditions for DPV were: pulse amplitude 50 mV, pulse width 0.5 s and scan rate 5 mV s^{-1}. All experiments were performed at room temperature (21 ± 2 ºC) in triplicate (n = 3) and the main electrolyte solution used was phosphate buffer solutions (PBS, pH 7.0).

DP voltammograms were background-subtracted and baseline-corrected. Plots of the voltammetric curves for final presentation in this study were drawn using Origin Pro 8® software (Northampton, MA, USA).

2.6. Pharmaceutical sample preparation

For pharmaceutical formulae assessment, tablets of paracetamol (labeled 500 mg / tablet, n = 10) were completely grinded and dissolved in ultrapure water.

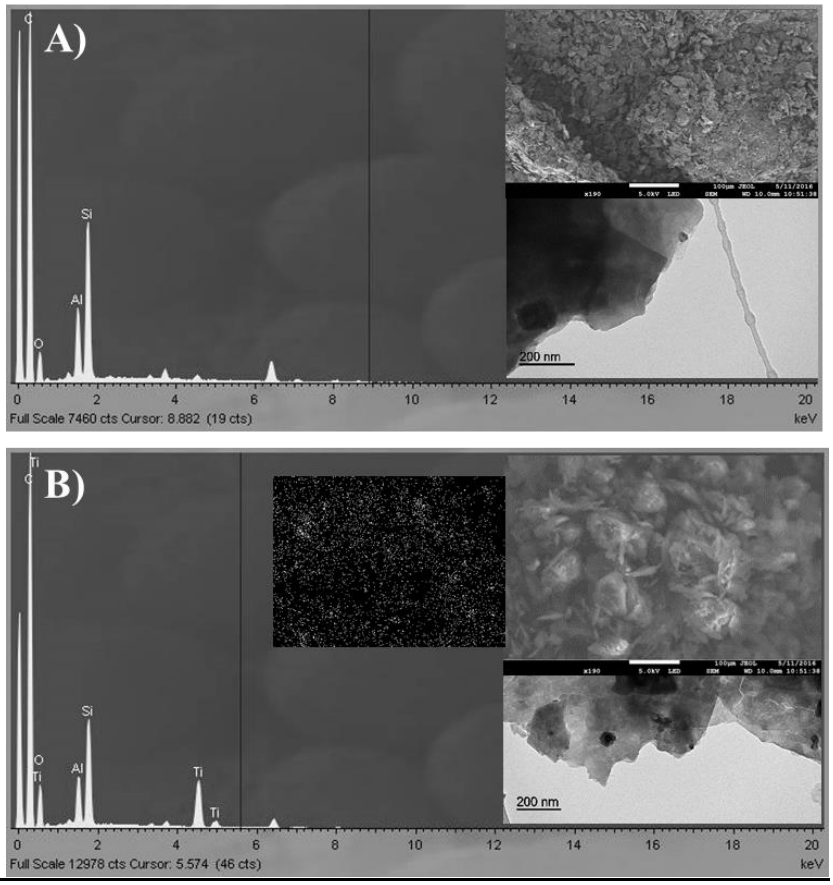
3. RESULTS AND DISCUSSION

3.1. Electrode characterization

TiO_2@C was characterized by means of EDX, SEM and TEM microscopy. The presence of Ti, was confirmed in EDX (Figure 1).

Nano sized dimension and dispersion grade were observed at figure 1B. Comparing SEM and TEM images from figure 1A and 1B, it can be noticed that graphite powder was nanostructured with titanium successfully. Moreover, SEM images also confirm almost identical features of graphite powder material to the one employed in TiO_2@C. Such statement is further strengthened by almost identical morphology and particle size. Ti oxide presence did not significantly influence the structural and morphological properties of graphite powder. However, metal oxide certainly altered electrodic

properties, as further explored in this work. Furthermore, the metal oxide was seemingly adsorbed on the carbon reticule, which is in accordance to other works involving adsorption and chemosorption of metals on carbon based matrices [11,12,13].



Material	% Ti	% C	% O	% Si	% Al
Pencil Carbon powder	-	76.10	15.19	6.16	2.54
TiO2@C	4.77	68.70	20.50	4.24	1.80

Figure 1. EDX spectrum with SEM TEM imagens: **A**) unmodified pencil powder **B)** TiO_2@C powder. In addition, table with EDX elementary results.

All herein studied electrodes were also evaluated by means of EIS, due to its suitability in providing data regarding electron transfer kinetics in electrochemical systems [15,16,7,8].

The novel biosensor electrochemical characterization is shown in figure 2, where a comparison can be stated between it and the other tested sensors. In addition, a Randles electric equivalence circuit model was proposed for Lcc-TiO_2@CPE.

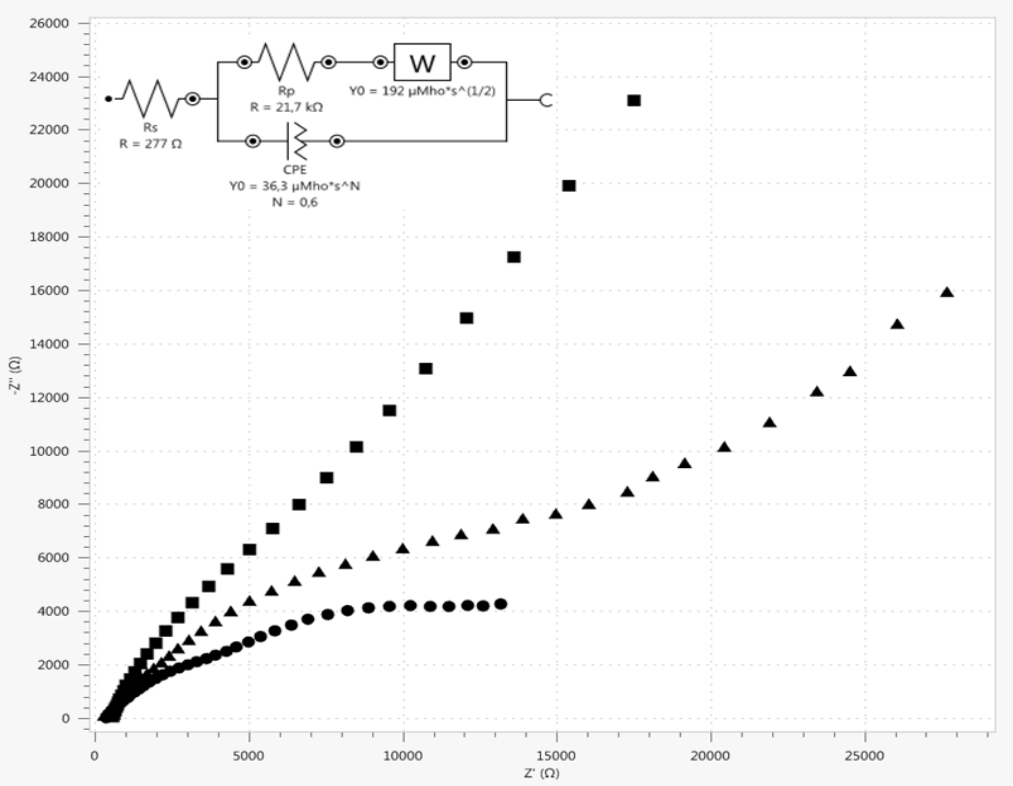


Figure 2. Nyquist plots obtained for: (squares) CPE, (triangles) Lcc-TiO_2@CPE and (circles) TiO_2@CPE. **Insert.** Equivalent electrical circuit for Lcc-TiO_2@CPE, presenting the solution resistance ($_{Rs}$), the polarization resistance (R_p), the Warburg impedance (W) and the capacitance of the system (Constant Phase Element = CPE).

It was observed that TiO_2@CPE matrix exhibited better results concerning electron transfer than CPE, whereas Lcc-TiO_2@CPE presented intermediary profile. Moreover, electrolyte resistance values (Rs), polarization resistance (Rp), Warburg diffusion (W) and system capacitance for Lcc-TiO_2@CPE where bellow to those of CPE electrode (Data not shown). This may be likely attributed to a lower charge transfer resistance and a higher conductivity and diffusion. Moreover, the pseudo-capacitance as well as frequency independent taken from the constant phase element of each circuit attested a capacitive behavior to all electrodes, which is in consonance to literature data, as solid electrodes tend to present a capacitive behavior, with frequency independent values next to 1 [17,18,19,20]. Therefore, TiO_2@CPE and Lcc-TiO_2@CPE are more efficient to provide electron transfer on electrode-electrolyte interface.

3.2. Electro-catalytic response

Lcc-TiO_2@CPE was primarily evaluated for phenolic detection using catechol. The results were compared between the studied electrodes, namely: TiO_2@CPE, Lcc-TiO_2@CPE, and CPE (Fig. 3).

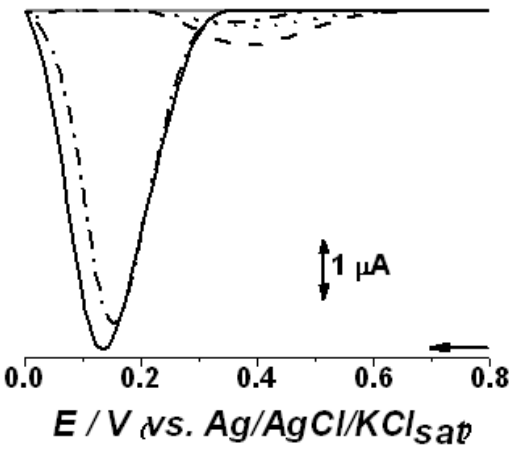


Figure 3. DP voltammograms obtained for 100 µM catechol solution at CPE (•••), TiO_2@CPE (−−−), Lcc@CPE (−▪−) and Lcc-TiO_2@CPE (▬▬) all in 0.1 M PBS (pH 7.0).

In order to check the synergistic effect of polyphenoloxidase in the sensor performance for phenolic detection, the analysis was carried out in order to express mainly the increase in sensibility.

Lcc-TiO_2@CPE presented highest sensibility when compared to other sensors. Furthermore, the biosensor presented higher selectivity for phenolic compounds (Fig.3) as also observed in a previous study [16]. Nevertheless, TiO_2@CPE also presented high sensibility, which can be implied to occur due to electro-catalysis. This electro-catalytic effect is well reported in literature, and is common to electrodes whose matrices contemplate transition metals such as Ti [11,12,17].

A reduction process occurred at peak potential, E_{pc}= 0.14 V, which correspond to a cathodic shift of 0.26 V (vs. Ag/AgCl) when compared to the sensor TiO_2@CPE (Fig.3). Henceforth, TiO_2@CPE and Lcc-TiO_2@CPE were also evaluated for paracetamol assessment.

3.3. Biosensor application in drugs detection

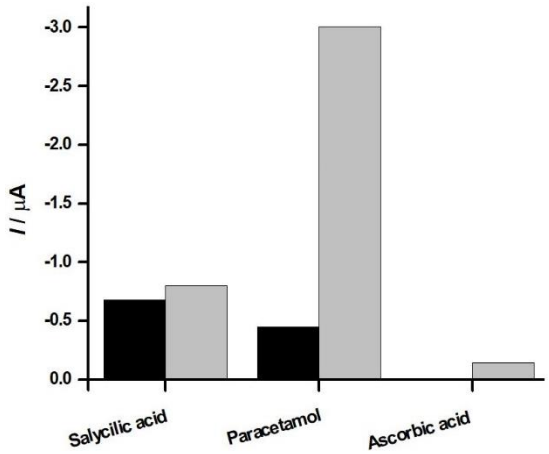


Figure 4. Response to TiO_2@CPE (black) and Lcc-TiO_2@CPE (light grey) in the analysis of different analytes, at 100 µM concentration in 0.1 M PBS (pH 7.0).

Lcc-TiO_2@CPE and TiO_2@CPE were tested for different phenolic drugs other than paracetamol, in order to assess its response, results are displayed in Figure 4.

Results indicate that TiO_2@CPE performance was superior than that of CPE, whereas Lcc-TiO_2@CPE presented remarkable response for paracetamol. In order to ensure that the enhanced response is related to bio-catalytic activity rather than electro-catalytic effect, a non phenolic compound (ascorbic acid) was also evaluated (Figure 4).

Although the oxidation product of ascorbic acid has similar reduction potential to the one of paracetamol, the observed response was far inferior than that of this phenolic analgesic. Moreover, it is noteworthy to mention the propriety of salicylic acid detection, a methyl salycilate degradation product. Such results indicate prospective uses for our novel biosensor.

In order to check the analytical performance of the biosensor a pH response study was performed for paracetamol (Fig. 5).

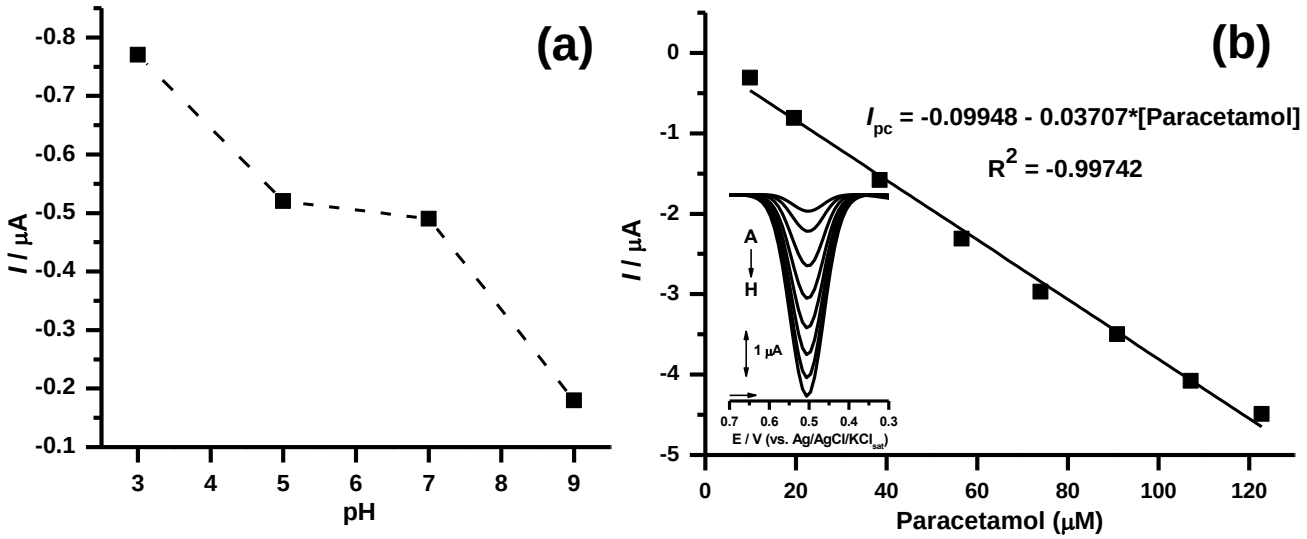


Figure 5. (a) pH response study for Lcc-TiO_2@CPE. **(b)** Calibration curve obtained from DPVs in 0.1 M PBS (pH 3.0), for increasing concentrations (A) 10 to (H) of paracetamol.

Results evidence that paracetamol presented high response either at acid or mild acid pH 3.0. The pH of saturated paracetamol aqueous solution was 6.0, and its aqueous solubility at 25°C was 14 mg/mL. Therefore, the pH needed for the best response is consistent with the chemical characteristics of this compound and imply moreover the occurrence of a proton transfer. Since assays take instants to perform, such acid environment may not promote prompt paracetamol hydrolysis, henceforth justifying the observed results.

From these results, calibration plots for paracetamol were performed using Lcc-TiO_2@CPE in its respective optimum pH condition (Fig. 5b). The linear range from 10 to 120 µM was obtained for paracetamol (I_{pc} = -0.09948 – 0.03707*[paracetamol]; (R^2 = 0.99742) at Lcc-TiO_2@CPE (Fig.5b).

Thus, the calibration curve presented good linearity and the limit of detection (LoD) calculated from it was of 1 µM.

The table 2 displays a comparison between the analytical performance of Lcc-TiO_2@CPE, and those of other systems described in literature for paracetamol detection (Table 2), henceforth evidencing that the herein proposed biosensor presents higher sensibility.

Table 2. Comparison of results obtained for paracetamol detection with different electrodes.

Electrode	Methods	LoD (µM)	Linear range (µM)	Reference
Lcc-TiO_2@CPE	$DPV_{reduction}$	1	10–120	This work
Au/PANI-cMWCNT/*Bacillus sp.*/GA	Amperometric	2.9	5 – 630	[13]
D.O.-probemembrane/ Lcc-gelatin /GA	Amperometric	-	2–15	[3]
CPE/Eggplant-PPO	$DPV_{reduction}$	5	60-115	[17]

CPE = carbon paste electrode; cMWCNT = carboxylated multiwalled carbonnanotube; PANI = polyaniline; GA = glutaraldehyde; Lcc = laccase; D.O. = dissolved oxygen; PPO = polyphenoloxidase; LoD = limit of detection.

3.4. Biosensor suitability for commercial samples

Table 3 evidence the results concerning the determination of paracetamol in commercial pharmaceutical formulations (tablets) using the proposed DPV method (Table 3).

Table 3. Results obtained in the determination of paracetamol in commercial pharmaceutical formulations (tablets) using the proposed DPV method.

Sensor	Sample	Theoretical concentration (mg/tablet)	Experimental concentration (mg) ± SD[a]	Mean recovery (%) ± SD[a]
Lcc-TiO_2@CPE	Paracetamol tablet	500	485.75 (± 0.92)	97.15% (± 0.18)

[a]SD: Standard deviation of three replicate determinations, average of three replicate determinations.

As evidenced in table 3, Lcc-TiO_2@CPE presented good recovery, which was near 100%, and the relative standard deviation was lower than 5% (n = 3), henceforth evidencing accuracy and precision.

Our findings showed that the excipients do not interfere in the analysis, reinforcing the selectivity of the Lcc-TiO_2@CPE towards complex pharmaceutical formulations. Nonetheless, pharmaceutical excipients are known to not be electro-active, which in turn, corroborate to the results herein seen.

Therefore, the Lcc-TiO_2@CPE biosensor is a promising alternative tool to determine the concentration of phenolic analytes such as paracetamol in drug samples [1,2].

4. CONCLUSION

The novel biosensor herein presented is a low cost and versatile alternative for paracetamol assessment in pharmaceutical samples, since it exhibited good analytic performance, as well as LoD of 1 μM, which was nine-fold superior than the one observed for unmodified carbon paste sensor.

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